Quantitation of Amoxicillin in Urine by Nuclear Magnetic Resonance. Application to Five Cases

İdrardaki Amoksisilinin Nukleer Magnetik Rezonans ile Miktarının Tayini. Beş Durum için Uygulama

Amoxicillin Quantitation in Urine

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Özet

Amaç: Bu makalede idrardaki amoksisilinin analiz ve miktar tayini için numunelerde minimum bir ön hazırlık ile nukleer magnetik rezonans (NMR) ile tayini için bir prosedür öneriyoruz. Beş adet klinik örneği analiz edildi, her örnekte amoksisilinin teşhisinden sonra miktarı tayin edildi. Gereç ve Yöntem: Amoksisilin kullananların idrar örnekleri IRB ce kabul edilmiş protokole göre toplandı. Donörlerden idrar alınış tarihleri, tedavi süreleri ve yazılmış ilaç miktarlarını kaydetmeleri istendi. Toplanmış örnekler derhal -20 0C de deneyler için gerekinceye kadar donduruldu. Suyun bastırılması "Watersculp" tekniği bütün örnekler için kullanıldı. Hızlı bir ön asit uygulaması idrar numunelerinde NMR spektrumunda üre sinyalini yok etti. Eklenen numune örnekleri ve amoksisilin'in seçilmiş sinyallerinin integrasyonu ile bir kalibrasyon eğrisi elde edildi. Bu metot ile 0.01- 1mg/mL aralığında (korelasyon sabiti 0.995) linear cevap elde edilmiştir. Bulgular: Klinikden alınan örneklerdeki amoksisilin miktarları tayin edilmiştir. İdrardaki ilaç derişimi0.517 mg/mL ile 0.326 mg/mL aralığında değişmiştir. Tartışma: İnsan idrarındaki amoksisilin tayini için NMR araştırmalarının üstünlük ve güvenirliğinin araştırılması tartışılmıştır

Anahtar Kelimeler

Amoxicillin; NMR Spectroscopy; İdrar

Abstract

Aim: In this paper, we propose a procedure for the analysis and quantification of amoxicillin in urine with minimum pre-treatment of the samples using Nuclear Magnetic Resonance spectroscopy (NMR). Five clinical samples were analyzed and amoxicillin was detected and quantified in each case. Material and Method: Samples of urine from amoxicillin users were collected in accordance with an IRB-approved protocol. Donors were asked to record the day of urine collection, the length of treatment and the amount of drug prescribed. Samples were collected and immediately frozen at -20°C until required for experiments. The water suppression technique "Watersculpt" was used in all samples. A rapid acidic pre-treatment eliminated the urea signal from the NMR spectra of urine samples. A calibration curve was obtained using spiked samples and by integration of selected signals from amoxicillin. The method gave a linear response (correlation coefficient of 0.995) over the range 0.01-1 mg/mL. Results: Subsequent quantitation of the amount of amoxicillin present in samples from clinical cases was performed. The concentration of the drug in urine varied from 0.517 mg/mL to 0.326 mg/mL. Discussion: The benefit and reliability of NMR investigations of human urine for the detection of amoxicillin are discussed.

Keywords

Amoxicillin; NMR Spectroscopy; Urine

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Introduction

Amoxicillin ((2S,5R,6R)-6-[[(2R)-amino-2(4-hydroxyphenyl) acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid) is a β-lactam antibiotic commonly prescribed by doctors to treat various infections. It is frequently used in combination with other antibiotics depending on the type of infection and is a broad spectrum penicillin [1]. It may be administered in clinical settings of pneumonia, urinary tract infections and otitis among others [2-3].

Amoxicillin (Fig. 1) is directly obtained from 6-aminopenicillanic acid [4]. Its broad commercialization calls for new methodologies to determine its pharmacokinetics and for its detection in biofluids in case of intoxication/adverse reaction [5]. One of the most dangerous side effects of amoxicillin is anaphylactic reaction and can be lethal [5]. Methods have been developed and validated in order to determine amoxicillin in biofluids, mainly plasma [6-8]. However, no example of direct detection in urine with minimal pretreatment has been reported. A rapid, convenient method to detect amoxicillin in urine is all the more important to develop since urine is a biological matrix commonly tested for drugs and since 60 to 70% of amoxicillin is excreted unchanged in urine. Testing of urine is usually done in cases where drugs need to be identified, in postmortem analysis or to support or deny a person's testimonial [9]. Analytical laboratories generally use well accepted methods such as gas chromatography-mass spectrometry[10]. However these methods have certain downfalls. GC-MS and HPLC require sample preparation which, if not done correctly, can lead to sample loss and questionable results [11]. Sample preparation such as extraction and derivatization are very time consuming and require extensive training. Immunoassays are not confirmatory tests, require further testing and are subject to high rates of false negatives or false positives [12]. NMR spectroscopy can be used to analyze biological fluids for the diagnosis of acute poisoning and drug overdoses without the need for separation and/or derivatization steps [13]. NMR has been used to identify and quantitate levels of various xenobiotics causing poisoning: salicylate, valproic acid, paraquat, tetrahydrofuran and methanol and drugs of abuse such as GHB (gamma hydroxybutyric acid) and GBL (gamma hydroxybutyrolactone) in biofluids [14-15]. Acetaminophen and ibuprofen metabolites in urine have also been identified and studies on creatinine supplementation are found in the literature [16-17]. Finally, 3,4-methylenedioxymethamphetamine (MDMA/ecstasy) has been detected in urine by ^{1}H NMR in five cases of intoxication [18]. This research shows that NMR spectroscopy can be used to accurately quantitate drugs in urine with a high level of reproducibility. However, there has not been any study on the use of NMR to detect amoxicillin directly in urine samples. In this paper, we propose an NMR procedure for the analysis and quantification of amoxicillin in urine with minimum pre-treatment of the samples. Five clinical samples were analyzed and amoxicillin was detected and quantified in each case.

Materials and Method

Biological samples were collected and analyzed in New York city (USA). Collection occurred in the fall 2014, method development and analysis of samples took place in the fall 2014 and spring 2015.

Reagents

Reagents used included amoxicillin tablet (1g, Teva) and deuterium oxide (99.9% Cambridge Isotope).

Biological samples

Samples of urine from amoxicillin users were collected in accordance with an IRB (International Review Board) approved protocol. This study is in agreement with international guidelines about "Regulations on Pharmaceutical Research". Donors were recruited on a voluntary basis and remained anonymous. An informed consent document was signed in each case. Donors were asked to record the day of urine collection, the length of treatment and the amount of drug prescribed. There was no link identifying the donor with the sample. Samples were collected and immediately frozen at -20°C until required for experiments.

Proton NMR spectroscopy analysis and processing

NMR data were collected on a Bruker 500 MHz NMR spectrometer (Bruker BioSpin, Billerica, MA). Acquisition of spectra was carried out with Topspin 3.1 software. Processing was performed with MestReNova 8.3. The spectrometer transmitter was locked to D_2O and all the spectra were acquired at 298 K. Data were collected into 64k data points plus 2 dummy scans and the spectral window was 20ppm. All experiments were performed with automatic tune and match, z-axis gradient, and gradient shimming that was equipped in the instrument.

Water suppression techniques

One dimensional spectra were obtained using two different water suppression techniques: PURGE (Presaturation Utilizing Relaxation Gradients and Echoes) and Watersculpt [19-21]. Both methods (PURGE and Watersculpt) were compared for water suppression efficiency.

T1 calculations for quantitation experiments

In order to obtain an accuracy of 1% or less for quantitation with ^{1}H NMR, the recycle time (the time between pulses, d1) must be at least five times the T1 of the slowest relaxing signal of interest in the spectrum. Separate experiments were performed to determine T1 of protons b (δ=5.56) and c (δ=5.51) which were considered to establish the calibration curve. T1 calculations were done using TopSpin 3.1 software. A T1 value of 2.529 seconds (protons c, δ=5.51) was calculated.

Spectrum of amoxicillin in H_2O

The ^{1}H NMR spectrum of amoxicillin in water was recorded for proton assignments and is shown in figure 1. Chemical shifts multiplicity and coupling constants are listed in table 1.

pH Dependency experiments

Urine NMR samples at neutral pH (7) were acidified by addition of 10 µL of 12 M hydrochloric acid (pH 2) or brought to a basic pH (10) by addition of 10 µL of 6 M sodium hydroxide.

Urine sample preparation

Spiked samples: Urine from an anonymous donor (200 mL) was collected, divided into 10 mL aliquots and frozen at -15 °C until needed for further analysis. Amoxicillin (Teva) was used

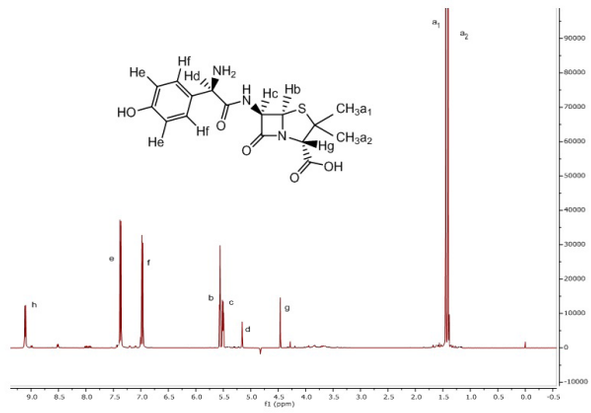

Figure 1. Spectrum of 10 mg/mL amoxicillin in H_2O (Watersculpt), 16 scans.

Table 1. ^1H NMR data of amoxicillin in water: Chemical shifts, coupling constants, number of protons, and peak multiplicity.

Proton	Chemical Shift (ppm)	J Coupling (Hz)	Number of protons	Multiplicity
a1	1.43		3	s
a2	1.41		3	s
g	4.46		1	s
d	5.16		1	s
c	5.51	J1 = 6.74	1	dd
		J2 = 3.93		
b	5.56	3.78	1	d
f	6.98	8.76	2	d
e	7.37	9.17	2	d
h	9.11	6.98		d

to spike the samples. Before each sample preparation, urine was warmed up in a water bath at 37 °C for 25 minutes. The needed amount of dry amoxicillin powder was weighed out and dissolved. Urine (500 µL) and 10 µL of 12 M hydrochloric acid were introduced into a 5 mm tube with a coaxial capillary tube containing a solution of 3-trimetyhylsilyl 2,2',3,3'-tetradeuteropropionic acid (TSP-d_4, 0.1mg/ mL) in D_2O providing an internal field frequency lock and reference (δ=0).

Clinical samples: Each urine sample (500 µL) was introduced into a 5 mm tube with 10 µL of 12 M hydrochloric acid and a coaxial capillary tube containing TSP-d4 (0.1mg/ mL in D2O).

Calibration curve

Spiked samples of 1.00, 0.75, 0.5, 0.25, 0.1, and 0.01 mg/mL of dry amoxicillin in blank urine were analyzed. Concentrations of 1.00 to 0.25 mg/mL were scanned 128 times. Concentrations of 0.1 mg/mL were scanned 256 times. Concentrations of 0.01 mg/mL were scanned 3248 times. Each concentration was analyzed in triplicate and the average integration of the signals of interest (protons b and c) was calculated to generate the calibration curve.

Standard deviation, limit of detection and limit of quantitation

The standard deviation was determined by running seven independent experiments with a concentration of 0.05 mg/mL. Statistical analysis of the seven spiked urine samples was performed based on the area of the peaks of interest vs. the area of the internal standard peak. Relative standard deviation was 0.36%. The very nature of NMR implies that the LOD depends on the amount of scans used for the experiment. In this study, the highest amount of scans used was 3248 which allows for a LOQ of 0.01 mg/mL

Results

Choice of optimal conditions for NMR analysis of amoxicillin in urine

^1H NMR experiments in aqueous bio-fluids require the use of a water suppression technique. We first tested two different water suppression methods: Purge and Watersculpt. NMR spectra of a 2mg/mL amoxicillin in urine sample were recorded and compared. The water signal was clearly better suppressed using Watersculpt and we chose this water suppression technique for subsequent experiments. Next, we investigated the best amoxicillin signals to select for the quantitation of the drug in urine. The NMR spectrum of blank urine is crowded with many peaks from urine metabolites. The region between 4.5-5.5 ppm and 6.0 to 6.5 ppm is where signals from exogenous drugs are most likely to be detected. In addition, the signals selected for quantitation must be as far as possible from the suppressed water signal (4.79 ppm) to avoid any base line distortion. These conditions preclude protons a, d, e, f, g and h -table 1-. On the other hand, signals from protons b and c appear in the right area. However, at low concentration, the broad urea peak at 5.7 ppm interferes with the detection of signals b and c. We decided to see if a pH induced shift of the urea signal would lead to better signal separation. No shift was observed in basic conditions. Fortunately, in acidic conditions the urea peak disappears and no longer overlaps with the amoxicillin peaks (Fig. 2). Urea reacts with hydrochloric acid to form an ammonium salt (NH_4+Cl-). The ammonium nucleus is in a very symmetrical environment resulting in a small quadrupole moment. In this case, ^1H-^{14}N coupling is observed and the N-H signal is split into three sharp peaks around 7 ppm due to the three possible spin states of nitrogen (+1, 0, -1) [22]. The urea's broad peak around 5.7 ppm is no longer observed and this allows the accurate integration of protons b and c. We therefore decided to use acidic conditions and to select protons b and c for quantitation.

Once the optimal conditions were determined, a calibration curve was established with spiked urine samples by integrating the area from protons b and c signals at different concentrations -materials and methods, section 2.9, Calibration curve-.

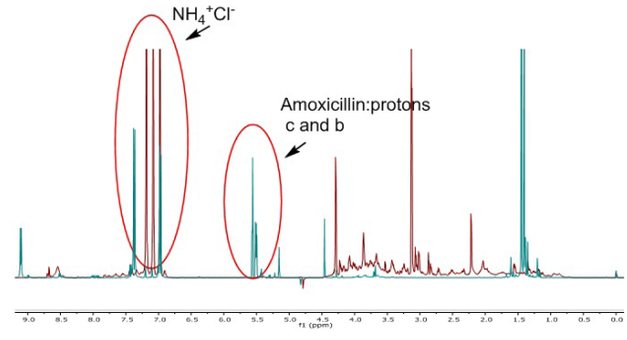

Figure 2. Overlay of the spectra of amoxicillin (green) and urine (red) at pH 2.

Application of the NMR method to five clinical cases

Five samples of urine donated by volunteers were analyzed in triplicate. The presence of amoxicillin was based on the observed peak pattern in the 5-6 ppm region (Fig.3). This region is clear of other urine metabolites. The presence of the peaks from the methyl protons (Ha1 δ=1.41 and Ha2 δ=1.43) the singlets Hg (δ=4.46) and Hd (δ=5.16) and the aromatic protons (He δ=7.37 and Hf δ=6.98) of amoxicillin in the spectra confirmed the results. Quantitative analysis was performed by using the calibration curve previously established. Table 2 summarizes the results of the analysis.

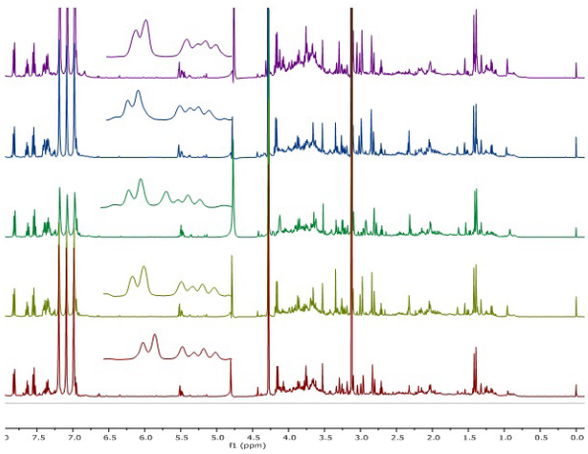

Figure 3. Overlay of five urine samples from amoxicillin users. Spectra are organized in decreasing order from sample 1, top, to sample 5, bottom. Insert zooms show integrated signals for protons c and b.

Table 2. Summary of the five clinical cases.

Sample	Amoxicillin ingested	Collection date	Concentration mg/mL
1	500 mg every 8 hours (4 days)	Day 2	0.492
2	500 mg every 8 hours (5 days)	Day 4	0.374
3	500 mg every 8 hours (4 days)	Day 3	0.517
4	500 mg every 8 hours (5 days)	Day 2	0.326
5	500 mg every 8 hours (4 days)	Day 3	0.432

Discussion

In all cases, NMR analysis of the samples confirmed the presence of amoxicillin -The presence of amoxicillin was also confirmed using HPLC [8]-. The concentration of the drug in urine varied from 0.517 mg/mL to 0.326 mg/mL. Amoxicillin is stable in gastric acid and rapidly absorbed from the gut to an extent of 72 to 93%. Absorption is independent of food intake. Approximately 60 to 70% of Amoxicillin is excreted unchanged in urine during the first 6 hours after administration of a standard dose. For all samples the expected concentration of amoxicillin found in urine should be between 0.324 mg/mL and 0.488 mg/mL (for an uptake between 72 to 93%; an excretion between 60 to 70% and an average of 2000 mL of urine produced per day). The amounts found in the urine of the five volunteers vary between 0.326 and 0.517 mg/mL. Our results in table 2 are close to the range of expected concentrations. In any case, the subjects' metabolic rates, along with the amount of fluid consumed would affect amounts excreted, which may explain the variations observed. These results suggest that ^{1}H NMR spectroscopy could provide a convenient tool for the rapid detection of amoxicillin in human urine. The method presented here offers the advantage of a rapid diagnosis with little urine needed and virtually no sample preparation. The only manipulation of the urine sample is a simple addition of acid directly into the NMR tube (10 µL of 12 M hydrochloric). Furthermore, in the concentration range studied, quantitative data can be collected and samples were analyzed within 20 minutes. In an emergency context, the NMR method would offer a rapid identification procedure for amoxicillin. In addition, the NMR method is also a non-destructive method which makes it a valuable tool in forensics. Finally, all NMR-active compounds in urine can be detected at once, meaning that the method can be used to detect multiple analytes in one single experiment. The limitation of using NMR is that at lower concentrations, the quantification procedure can be difficult due to the amount of scans needed. This may extend the time of analysis. Alternatively, a stronger magnet may be used.

Conclusion

We successfully used ^{1}H NMR to detect and quantify amoxicillin in the urine of five patients. Samples had minimal manipulation i.e., a small addition of acid directly into the NMR tube, which makes the NMR method developed very appealing. NMR spectroscopy provides an advantage in both forensic and clinical cases when a quick identification is needed. In an emergency setting, it can provide a fast identification and confirmatory technique. In forensic cases this technique is also advantageous because it is non-destructive. One of the disadvantages of NMR however is that at lower concentrations it can be harder to detect the drug. More scans may be required, or a more sensitive NMR instrument -stronger magnet- may be needed. Future research to explore NMR spectroscopy for the detection of amoxicillin will include analyses at lower concentrations and drug-drug interactions examination. The presence of other drugs can potentially induce chemical shits and this warrants further investigation.

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Competing interests

The authors declare that they have no competing interests.

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